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Mice Deficient in Biglycan and Fibromodulin as a Model for Temporomandibular Joint Osteoarthritis

Sunil Wadhwa[a, b] Mildred Embree[a] Laurent Ameye[c] Marian F. Young[a]

[a]Craniofacial and Skeletal Diseases Branch, National Institutes of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Md., and [b]Department of Orthodontics, School of Dental Medicine, University of Connecticut Health Center, Farmington, Conn., USA; [c]Nestle Research Center, Department of Nutrition, Lausanne, Switzerland

Key Words
Proteoglycans · Biglycan · Fibromodulin · Temporomandibular joint · Osteoarthritis

Abstract
The temporomandibular joint (TMJ) within the craniofacial complex is unique. In humans, the TMJ can become diseased resulting in severe and disabling pain. There are no cures for TMJ disease at this time. Animal models of TMJ disease are scarce, but some exist, and they are described in this paper. We present in greater detail one animal model that is deficient in two extracellular matrix (ECM) proteoglycans, biglycan (BGN) and fibromodulin (FMOD). Doubly deficient BGN/FMOD mice develop premature TMJ osteoarthritis (OA). In order to explore the mechanistic basis of TMJ-OA, tissues from the condyle of mutant mice were examined for their relative capacity to differentiate and undergo apoptosis. Our data show that there is a redistribution of the critical ECM protein, type II collagen, in mutant mice compared with controls. Mutant mice also have increased apoptosis of the chondrocytes embedded in the articular cartilage. We speculate that the overall imbalance in apoptosis may be the cellular basis for the abnormal production of structural ECM proteins. The abnormal production of the ECM could, in turn, lead to premature erosion and degradation of the articular surface resulting in TMJ-OA. These data underscore the importance of the ECM in controlling the structural integrity of the TMJ.

Introduction

It is estimated that 10–40% of the population aged between 18 and 45 years has symptoms or signs of temporomandibular joint (TMJ) disease. Approximately 10% have osteoarthritis (OA) of the TMJ. However, the diagnosis of TMJ-OA is usually made only in later stages of

Abbreviations used in this paper

BGN	biglycan
ECM	extracellular matrix
FMOD	fibromodulin
TGF-β	transforming growth factor-β
TMJ	temporomandibular joint
OA	osteoarthritis
SLRP	small leucine-rich proteoglycan

S.W. and M.E. have joint first authorship.

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Sunil Wadhwa, DDS, PhD
Division of Orthodontics, School of Dental Medicine
University of Connecticut Health Center
Farmington, CT 06030 (USA)
Tel. +1 860 679 4899, Fax +1 860 679 1920, E-Mail wadhwa@uchc.edu

the disease, and therefore, the number of people who have OA of the TMJ may be higher.

TMJ-OA seems to affect two different age groups and may have two different etiologies. The older patient population is estimated to have a 20–40% occurrence of TMJ-OA [Nannmark et al., 1990; Widmalm et al., 1994; Sato et al., 1996] and is very unlikely to seek treatment [Carlsson, 1999; Anastassaki and Magnusson, 2004]. On the other hand, patients under the age of 30 are more likely to seek treatment for TMJ disorders, and it has been estimated that 10–66% of those who seek treatment have OA of their TMJs [Ogus, 1979; Stewart and Standish, 1983; Wiberg and Wanman, 1998]. Currently, there are no biomarkers or effective treatments for TMJ-OA. Imaging techniques can be used to diagnose TMJ-OA but only after the joint has been subjected to irreversible damage, and by this point, treatment of TMJ-OA merely involves palliative relief of the symptoms. Due to the limited capacity of joints to regenerate, it would be beneficial to detect OA of the TMJ at an early stage in order to prevent permanent joint damage. Furthermore, because the biopsying of human pre-osteoarthritic TMJ samples is virtually impossible, generation of TMJ-OA animal models that undergo the entire disease process is necessary.

There are two types of animal models for TMJ-OA: one is induced by trauma, while the other is occurs naturally. Traumatic induction of TMJ-OA typically involves surgical manipulation [Tominaga et al., 2002], forced mouth opening [Fujisawa et al., 2003], or the administration of inflammatory mediators within the joint [Harper et al., 2001; Tominaga et al., 2002]. Unlike traumatic models of TMJ-OA, naturally occurring models cover the whole disease process, particularly the earlier stages. Because both genetic and environmental factors may be precisely defined and controlled, naturally occurring models hold the potential to reveal the molecular pathways that influence the degeneration of the TMJ.

Currently, there are four reported naturally occurring mouse models of TMJ-OA. The first model was described by Silbermann and Livne [1979] in a strain of mice in the Institute of Cancer Research. Male mice of this strain developed osteoarthritic lesions in their TMJs progressing with age [Silbermann and Livne,1979]. Evidence of TMJ-OA first appeared at 6 months and consisted of fibrillations along the articular surface and in deeper tissue regions, a marked loss of tissue cellularity, and a reduction in safranin-0 staining. The authors concluded that the degenerative changes of mandibular condylar cartilage started at the more superficial regions and then proceeded towards the inferior regions [Livne and Silbermann, 1986]. The same group later hypothesized that the combination of reduced sulfated glycosaminoglycan and the decreased number of cells in the superficial articular layer of the mandibular condylar cartilage resulted in tissue with an architecture that is less suited to withstanding stress and thus more prone to the development of osteoarthritic lesions [Livne, 1994].

The second published naturally occurring mouse model of TMJ-OA was found in Del1 mice. Del1 transgenic mice produce a mutated form of the human type II collagen gene *(Col2A1)* driven by the human collagen type II promoter, which induces mutant protein expression solely in cartilage. At 3 months, the Del1 transgenic mice developed subchondral cysts in the mandibular condyle and occasional clefting. The osteoarthritic lesions became progressively worse until finally, at 15 months of age, the mandibular condylar cartilage was almost completely resorbed [Rintala et al., 1997]. Interestingly, the presence of the mutated type II collagen did not affect the growth of the mandibular condyle cartilage. The hypertrophic zone of the mandibular condylar cartilage appeared normal or even enhanced in Del1 transgenic mice compared with wildtype controls. After the growth period, the mandibular condylar cartilage starts to function as an articular cartilage. The authors hypothesized that it is at this time that an intact network of type II collagen molecules is needed to provide structural strength to the condylar cartilage. The fact that the osteoarthritic lesions started after the active growth period supports this concept.

The third naturally occurring TMJ-OA mouse model has a loss-of-function mutation in the gene encoding the α_1-chain of type XI collagen (Cho mice). Heterozygote Cho mice show evidence of TMJ-OA by 3 months of age consisting of changes in proteoglycan staining in the superficial articular layer of the mandibular condylar cartilage. By 9 months of age, fibrillations extending across the entire surface of the mandibular articular cartilage become evident [Xu et al., 2003]. Changes in collagen fibril diameter and matrix metalloproteinase 3 production were observed in the knee joint of these mice; however, it is not known if similar changes occur in the TMJ.

In this paper, we describe in detail a mouse model unable to produce the two extracellular matrix (ECM) proteoglycans, biglycan (BGN) and fibromodulin (FMOD). We have previously shown that mice doubly deficient in BGN and FMOD develop OA of the knee [Ameye et al., 2002] and their TMJs [Wadhwa et al., 2005]. In the TMJ, osteoarthritic lesions first start to develop at 6 months

and worsen progressively. When the doubly deficient mice are 18 months old, there is almost complete destruction of their TMJs. Our model is similar to the other naturally occurring mouse models of TMJ OA: (1) the osteoarthritic lesions do not develop until after the active growth period, and (2) the destruction of the TMJ complex progresses with age.

Naturally occurring mouse models of TMJ-OA are similar to the human form of the disease in that it usually occurs after the growth period; however, the progression may be different. Researchers have postulated that human TMJ-OA follows three stages. In the first stage, there is clicking of the joint and periodic locking, which are also common signs in other TMJ disorders. In the second stage, the TMJ becomes painful both at rest and during function. In the third stage, there is a reduction in clinical symptoms and normalization of function; however, radiographically, there is an increase in TMJ deformation [Zarb and Carlsson, 1999]. Since the evaluation of the signs and symptoms of TMJ-OA has not been assessed and is extremely difficult to perform in the naturally occurring mouse models, we do not know if the progression of TMJ-OA is similar to the human form of the disease.

BGN and FMOD are members of the small leucine repeat proteoglycan family (SLRP). Members of this family are characterized by having a small protein core, which consists predominantly of leucine-rich repeats. There are 13 known members that are divided into three classes (I, II and III) depending on their genomic organization and the similarity of their amino acid sequences. BGN belongs to the class I type and FMOD to the class II type of SLRP. Both proteoglycans are ubiquitously found in skeletal and connective tissues [Iozzo, 1997, 1999; Hocking et al., 1998; Ameye and Young, 2002; Wadhwa et al., 2004]. In this paper, we show that several cellular events were changed during the progression of TMJ-OA. They included altered expression of type II collagen and increased levels of apoptosis. These data reveal some of the molecular events occurring in the ECM that may regulate TMJ integrity and function.

Material and Methods

Generation of BGN and FMOD Single- and Double-Deficient Mice

All experiments were performed under an institutionally approved protocol for the use of animals in research (NIDCR-DIR-98-058 and 01-151). Mice deficient in BGN or FMOD were generated as described previously [Ameye et al., 2002]. Heterozygous BGN/FMOD-deficient mice were produced by breeding a homozygous BGN-deficient female *(bgn–/–/fmod+/+)* with a FMOD-deficient male *(bgn+/0/fmod–/–)*; BGN males are designed as *bgn–/0* since the *bgn* gene is located on the X chromosome and absent from the Y chromosome. F2 BGN/FMOD double-deficient (male *bgn–/0/fmod–/–*) mice were obtained by interbreeding F1 heterozygous BGN/FMOD mice.

Genotyping

All mice were genotyped for *bgn* and *fmod* alleles by polymerase chain reaction analysis as described previously [Ameye et al., 2002]. Polymerase chain reaction products were resolved by electrophoresis through 1.8% agarose gels, yielding bands of 212 bp for the wildtype *bgn* allele, 310 bp for the disrupted *bgn* allele, 280 bp for the wildtype *fmod* allele, and 603 bp for the disrupted *fmod* allele.

Histology

Wildtype and mutant mice were collected for histology at 3, 6, and 18 months. Whole mouse heads were sectioned into two halves and fixed in 10% formalin for 2 weeks at room temperature. The samples were washed with tap water for 5 min and decalcified in formic acid bone decalcifier solution (Immunocal, Decal Corporation, Tallman, N.Y., USA) for 4 weeks. Following decalcification, the heads were washed in tap water for 5 min and fixed for an additional 3 days in buffered zinc formalin (Z-fix, Anatech Ltd., Battle Creek, Mich., USA). Subsequently, the samples were processed for standard paraffin embedding and cut in sagittal sections.

Immunohistochemistry

Tissue sections were deparaffinized, rehydrated in graded ethanol series, and treated with 3% peroxide in methanol. The sections were digested for 1 h with 0.015 U/ml chondroitinase ABC (cat No. KE01502, Seikagaku Corp., Tokyo, Japan), rinsed in phosphate-buffered saline, and subsequently digested for 10 min with pepsin (Pepsin reagent™ for unmasking; Abcam). Immunohistochemistry staining was performed using the mouse broad-spectrum AEC kit (Histomouse Max, Zymed Laboratories Inc.) following the procedure recommended by the manufacturer. The expression for collagen type II was studied using primary antibody raised against mouse collagen type II antigen (1:100 in 10% goat serum; Chemicon, clone No. 8887). In order to evaluate for non-specific binding, substitution of the primary antibody with mouse immunoglobulin G was performed.

Labeling and Evaluation of Apoptotic Condylar Chondrocytes

Tissue sections from 3 wildtype mice and 3 mutant mice were deparaffinized and rehydrated in graded ethanol series. Condylar chondrocytes undergoing apoptosis were labeled using the Klenow FragEL DNA Fragmentation Kit (cat No. QIA21-1EA; Calbiochem) following the procedure recommended by the manufacturer. Images from the labeled tissue sections were captured using an AxioCam MR camera (Zeiss). Within a fixed area, the number of positively labeled condylar chondrocytes was counted and divided by the total number of cells. For each tissue section, three portions were analyzed, corresponding to anterior, center and posterior parts of the mandibular condylar cartilage.

Results

Type II Collagen Abnormal Expression in Normal Double Knockout Mice

At 3 months, in both wildtype and double-deficient mice, collagen type II expression was restricted to the inferior portions of the mandibular condylar cartilage and absent in the superficial layers (fig. 1). At 6 months of age, when the double-deficient mice first started to show histological evidence of TMJ-OA, we found that collagen type II expression lost its restricted localization and was expressed in the superficial layer of the condylar cartilage. On the other hand, at 6 months of age, collagen type II expression remained unchanged in the wildtype mice. This loss of the restriction of collagen type II expression did not become apparent until 18 months of age in the wildtype mice. Interestingly, at this age, wildtype mice also began to show histological evidence of TMJ-OA. Therefore, the loss of the restriction of collagen type II expression in both wildtype and double-deficient mice appears to be a biomarker of osteoarthritic degeneration of the condylar cartilage.

Apoptosis Levels in the Condyle of the Normal and Mutant Mice

FragEL labeling of cells undergoing programmed cell death (apoptosis) revealed an increase in the number of apoptotic positive cells from the double-deficient mice compared with the wildtype mice (fig. 2a, b). Examination of FragEL immunostaining in 10 sections from 2 different 3-month-old double knockout mice and 10 sections from 2 different 3-month-old wildtype mice revealed a 2-fold increase in the percentage of apoptotic cells in the mandibular condylar cartilage from the mutant mice compared with the wildtype mice (fig. 2d).

Discussion

Changes in the expression and localization of collagen type II in the mandibular condylar cartilage during OA could be due to a number of different mechanisms. One possible mechanism is that during the development of TMJ-OA, there is an increase in apoptosis of cells of the mandibular condylar cartilage that do not express collagen type II, as well as an increase in proliferation of chondroblastic cells on the surface that do express collagen type II. The fact that we have shown that there is an increase in apoptosis in the mandibular condylar cartilage from 3-month-old BGN/FMOD double-deficient mice compared with age-matched wildtype mice (fig. 2) and that there is an increase in proliferation in the mandibular condylar cartilage at 6 months in the doubly deficient mice [Wadhwa et al., 2005] supports this concept. Another possible mechanism which could be causing the expression of collagen type II in the articular layer of the mandibular condylar cartilage during TMJ-OA is total erosion of the superficial articular layer. We do not think this is likely, because at 3 months, there was no histological evidence of erosion in the mandibular condylar cartilage from double-deficient mice [Wadhwa et al., 2005]. Furthermore, at 6 months, when we started to see the uncharacteristic expression of collagen type II in the mandibular condylar cartilage from the double-deficient mice, there were only small vertical clefts, which did not extend to more than one quarter of the total thickness of the mandibular condylar cartilage.

Differences in the Progression of OA in the Knee and TMJ in the BGN/FMOD Double Knockout Mice

In this paper, we have shown that mice deficient in the ECM proteins BGN and FMOD develop TMJ-OA at 6 months, and by 18 months of age, there was almost complete destruction of the joint. Interestingly, in the knees of the same double-deficient mice, we found that OA first appeared as early as 1–3 months, and by 6 months, there was almost complete destruction of the joint [Ameye et al., 2002].

We propose that the differences between the progression of OA in these two tissues may be due to the differences in the anatomy and structure of the joints.

Unlike the articular cartilage of the knee, the cartilage of the mandibular condyle is considered a secondary cartilage in structure, function and development [Symons, 1965; Shen and Darendeliler, 2005]. Furthermore, the mandibular condylar cartilage is composed of fibrocartilage rather than hyaline cartilage [for a review, see Benjamin and Ralphs, 2004]. One of the unique properties of fibrocartilage is that it contains both type I and type II collagen compared with articular hyaline cartilage, which only contains type II collagen [Mizoguchi et al., 1992].

Additionally, unlike the articular cartilage of the knee, the mandibular condylar cartilage contains a growth plate during the active growth period. The growth plate is unique compared with other growth plates because it does not have linear columns of daughter cells (fig. 3). This nonlinear arrangement of daughter cells allows the mandibular condylar cartilage to have a multidirectional proliferative capacity. Therefore, unlike the growth plate of long bones, which can only expand unidirectionally, the random arrangement of daughter cells in mandibular

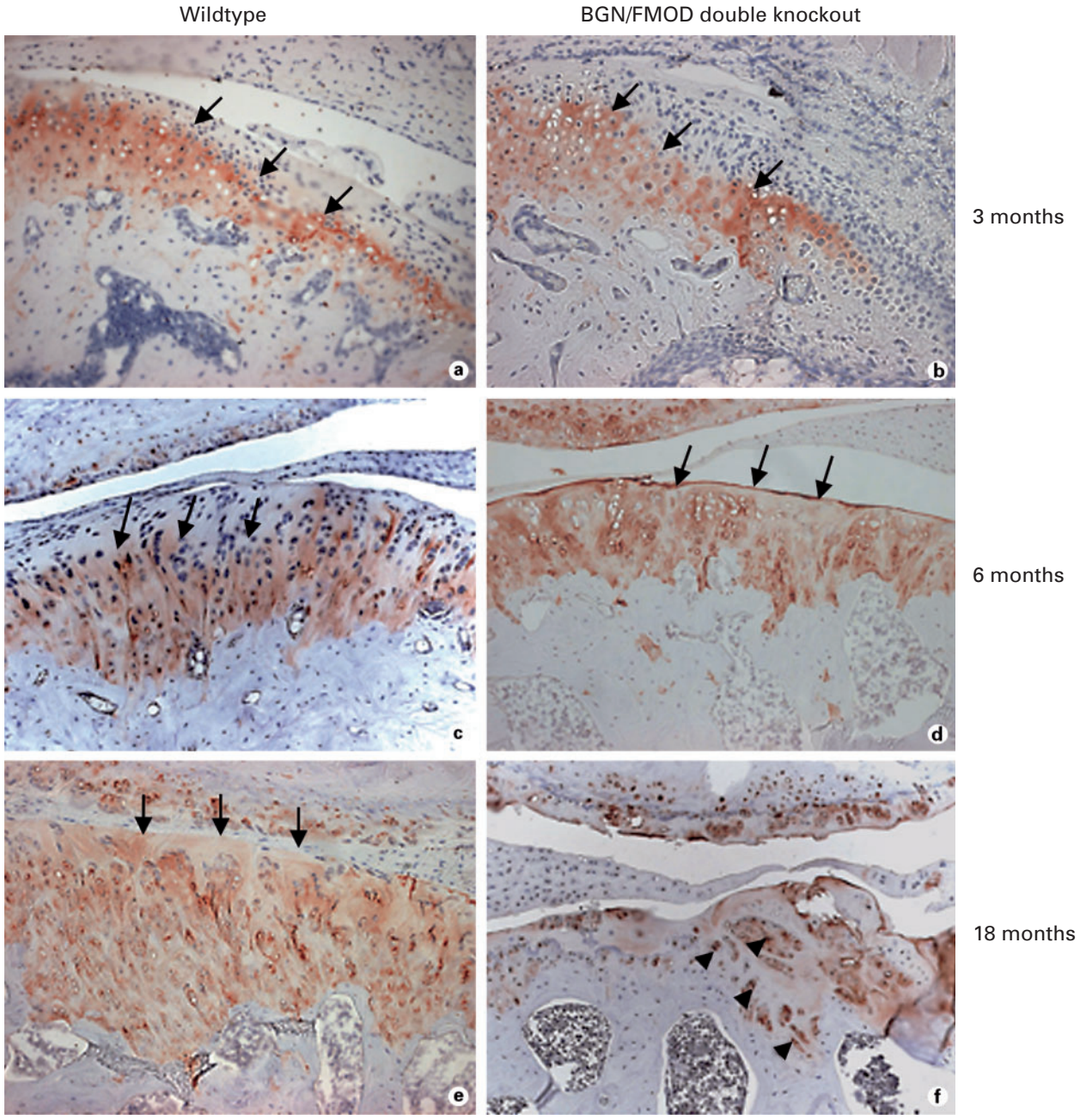
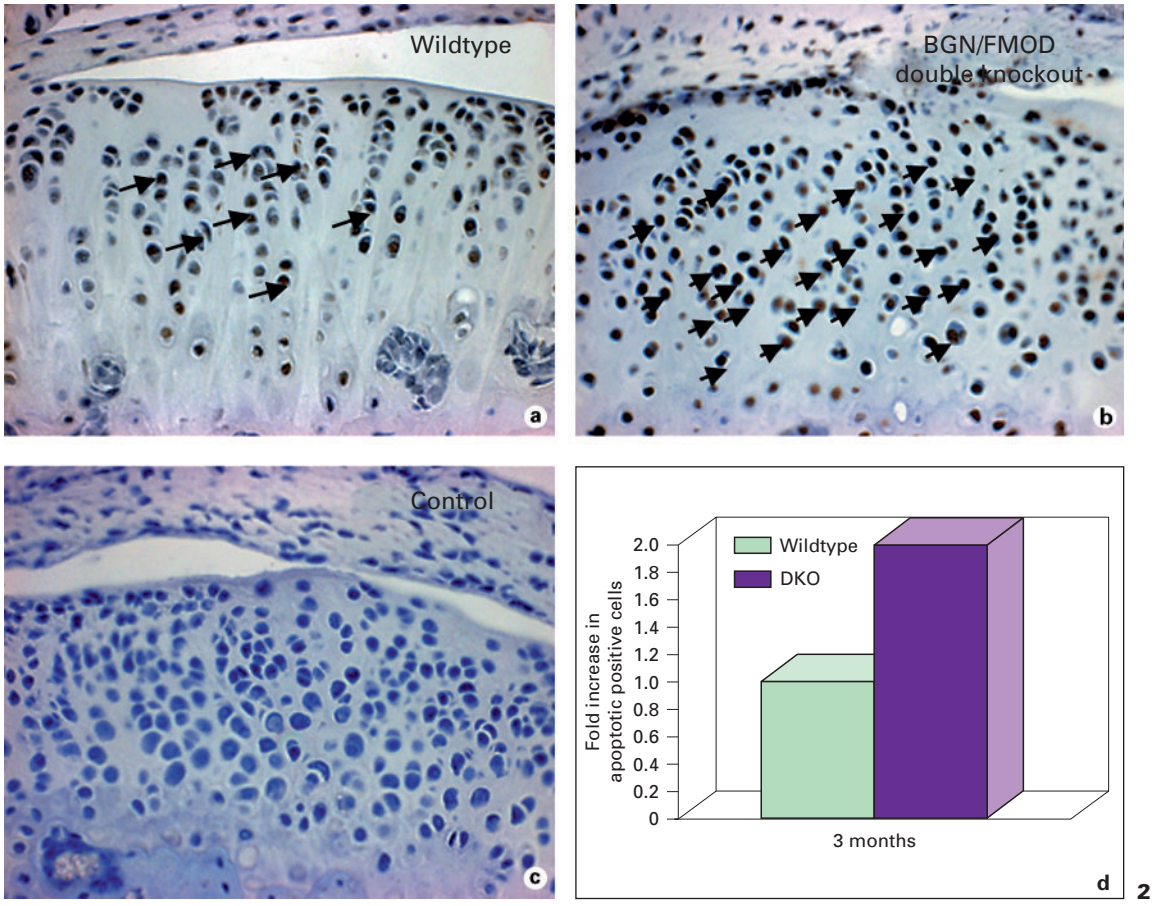


Fig. 1. Type II collagen immunohistochemistry (red) of wildtype (**a**, **c**, **e**) and BGN/FMOD double-deficient (**b**, **d**, **f**) mouse TMJ at 3, 6 and 18 months of age. Type II collagen localization is restricted to the inferior portions of the condylar cartilage in both wildtype (**a**) and BGN/FMOD double-deficient (**b**) mice at 3 months. At 6 months, type II collagen localization remains unchanged in wildtype mice (**c**), but becomes apparent in the superficial portion of the condylar cartilage in the BGN/FMOD double-deficient mice (**d**). Type II collagen expression does not become localized in the superficial portion of the condylar cartilage until 18 months in the wildtype mice (**e**). At this age, type II collagen expression increases in the BGN/FMOD double-deficient mice (**f**). Arrows indicate type II collagen immunostaining; arrowheads indicate intracellular type II collagen immunostaining.



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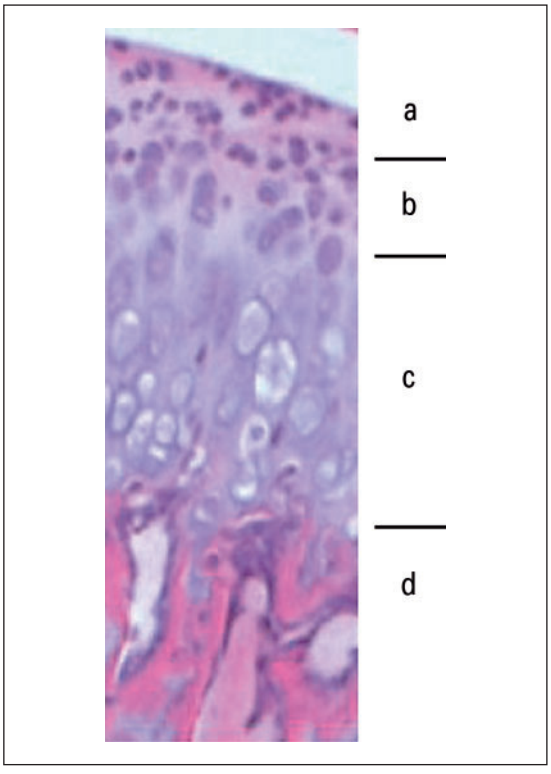

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Fig. 2. FragEL labeling (arrows) of cells in the mandibular condylar cartilage undergoing apoptosis in 3-month-old wildtype (**a**) and BGN/FMOD double-deficient (**b**) mice. Tissue sections treated with mouse immunoglobulin G served as a negative control (**c**). Apoptosis measured by FragEL labeling of condylar chondrocytes in wildtype and BGN/FMOD double-deficient mice. There was a 2-fold increase in cells undergoing apoptosis in the BGN/FMOD double-deficient mice compared with wildtype mice (**d**).

Fig. 3. Hematoxylin and eosin staining of a 49-day-old mandibular condylar cartilage. The mandibular condylar cartilage can be divided into four layers. **a** Superficial articular layer; **b** middle chondroblastic layer; **c** inferior hypertrophic layer; **d** subchondral bone. Note the non-linear arrangement of cells in the middle layer.

condylar cartilage allows growth in a multitude of directions [Enlow and Hans, 1996].

Because the TMJ and the knee are different, we suggest that the molecular basis for the development of disease may also be different. More specifically, it is possible that similar molecular pathways that are activated during OA cause a different outcome in the TMJ compared with the knee. For example, in both OA of the TMJ and OA of other joints, it has been reported that there is an increase in apoptosis [Aigner et al., 2004; Meng et al., 2005]. However, in the healthy mandibular condylar cartilage, there is a percentage of cells that normally undergo apoptosis [Huang et al., 2004]. In contrast, in healthy articular cartilage, there is almost a complete absence of cells undergoing apoptosis [Drissi et al., 2005]. In addition, the adult mandibular condylar cartilage has the ability to respond to growth factors by an increase in matrix synthesis and proliferation [Sviri et al., 2000], while the adult articular hyaline cartilage is believed to be unresponsive to growth factors. Therefore, we predict that an increase in apoptosis during OA in the knee would cause a more marked decrease in the cellularity of the articular cartilage compared with the fibrocartilage of the TMJ.

It has been reported that during development of OA in the knee, there is an increase in collagen type II expression [Salminen et al., 2001], and we have shown that there is also an increase in collagen type II expression during TMJ-OA. However, in contrast to the articular cartilage of other joints, the superficial layer of the mandibular condylar cartilage does not normally express type II collagen. The functional significance of the absence of collagen type II in the superficial layer of the mandibular cartilage is unknown, but we predict that the uncharacteristic expression of collagen type II in this layer during OA causes structural changes in the fibrocartilage of the TMJ, which differ from the increase in expression of collagen type II in hyaline articular cartilage.

Possible Role of BGN and FMOD

The exact cause by which the absence of BGN and FMOD accelerates the development of TMJ-OA is unknown. One possible explanation is that the absence of BGN and FMOD causes changes in the extracellular collagen network making the joint structures less suited to withstand physiologic mechanical loading. In support of this, we have found that in the knee of BGN/FMOD double-deficient mice, there were changes in the collagen fibril diameters compared with age-matched wildtype mice and mechanically compromised tendons. These changes were present before OA started to develop. In addition, increased mechanical loading by forced treadmill running caused an increased severity of OA in the knee [Ameye et al., 2002]. A similar mechanism could be taking place in the TMJ complex of double-deficient mice. A second possible explanation is that OA arises indirectly from changes in the underlying bone [Hayami et al., 2004]. Male mice who are deficient in BGN have been shown to have age-dependent osteoporosis [Xu et al., 1998]. However, whether there are changes in the bone of BGN/FMOD double-deficient mice is unknown. A third possible explanation is that changes in the TMJ fibrocartilage arise from the ability of BGN and FMOD to modulate members of the transforming growth factor (TGF)-β superfamily, which has been shown previously [Hildebrand et al., 1994]. The binding of BGN and FMOD to members of the TGF-β family may regulate their activity by sequestering them into the ECM, thereby preventing their binding with the cellular receptors. Support of this hypothesis comes from the examination of another SLRP-deficient mouse. In the bone marrow stromal cells from BGN/decorin double-deficient mice, we found that there is an increase in TGF-β activity [Bi et al., 2005]. Interestingly, a recent report has identified an increase in the susceptibility to OA in individuals with polymorphisms in asporin (an SLRP family member). Thus, the authors speculate that the identified polymorphisms cause changes in TGF-β activity [Kizawa et al., 2005].

Conclusion

In summary, we present new information about a mouse model of TMJ-OA and provide details of how it compares with other genetic and acquired models of this disease. Our data specifically show that mice deficient in the ECM proteoglycans, BGN and FMOD, acquired TMJ-OA earlier and to a greater extent compared with wildtype controls. The cell and molecular basis appears to be a combination of increased apoptosis of affected tissue leading to abnormal expression of a key structural component, type II collagen. The difference in the development of OA in the condyle compared with the knee is attributed to the differences in anatomy and suggests that each tissue must be studied separately to understand the basis of OA.

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